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Prevalence, antibiotic resistance, and phylogenetic analysis of *Listeria monocytogenes* isolated from various sources in Egypt: fish, vegetables, and humans

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Abstract

Listeriosis is a severe infection caused by consuming food contaminated with *Listeria monocytogenes*, the primary cause of human listeriosis. This study aimed to identify *L. monocytogenes* phenotypically and genotypically using *inlA* and *hlyA* virulence genes, followed by DNA sequence analysis and antimicrobial sensitivity in food resources and human samples. 345 Samples were obtained randomly from different markets and supermarkets in Qena, Egypt, including 115 fish, 25 fish containers, 90 vegetables, 90 diarrheal samples, and 25 hand swabs from patients and fishermen, respectively. *L. monocytogenes* was confirmed in 11.30% (39/345) and 3.48% (12/345) of the examined samples using culture and conventional PCR methods, respectively. The frequencies of *L. monocytogenes* in fish, fish containers, vegetables, and humans were 8.70, 3.48; 20, 8; 18.89, 1.11 and 6.09, 4.35% by both methods, respectively, with a statistically significant difference. Although *L. monocytogenes* predominated in 5-17 and 31-43-year age groups, the age risk factor for patients was statistically insignificant from an epidemiological perspective. Higher incidences were found in females and urban areas 7.27 and 4.76% than in males and rural areas 0 and 3.70%, without a statistically significant difference. The *inlA* was identified in all isolates, but the *hlyA* was identified in 41.67%. The highest multiple antibiotic resistance (MAR) index 0.625 was found in a diarrheal swab; all collected isolates were completely resistant to ampicillin. Additionally, 25% of *L. monocytogenes* stains were multidrug-resistant. According to phylogenetic analysis, the local isolates obtained from samples of tilapia, catfish, fish containers, cauliflower, and humans shared plenty of similarities.

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Introduction

Food safety remains a growing concern globally; the incidence of foodborne diseases is rising, causing significant morbidity and mortality rates. Developing countries, particularly those in the continents of the Middle East and North Africa, face considerable challenges in managing these diseases (1). One of the particularly serious foodborne illnesses is listeriosis, brought about by the bacterium *Listeria monocytogenes* (2). The prevalence of listeriosis varies by country and can vary between 0.1 and 11.3 occurrences for every million people (3). Listeriosis outbreaks linked to food are frequent in Egypt, and sporadic disease cases persist in various countries, such as the USA in 2015 and England in 2020, where ice cream and food or a food environment were implicated, respectively (4). The

common, gram-positive, rod-shaped bacteria *Listeria monocytogenes* is a member of the *Listeriaceae* family, which currently comprises 20 different species of *Listeria* (5). Only two species, *L. ivanovii*, and *L. monocytogenes*, are known to be pathogenic. *L. ivanovii* is mostly associated with animal foodborne outbreaks and infrequently linked to human infections, while *L. monocytogenes* is the main cause of listeriosis in humans (6). *Listeria monocytogenes* can tolerate a wide range of environmental challenges, making it a significant burden for the global food sector and health agencies. It can thrive within a pH range of 4.3-9.6, although a neutral pH is optimal for growth (7). As a psychrotroph, the organism can survive within a range of 0.5-45°C; nevertheless, it prefers temperatures around 30-37°C (7). Additionally, the bacterium is capable of long-term survival in frozen food items (8). Moreover, *L. monocytogenes* can resist desiccation, acid, heat, and disinfectants by developing surface biofilms (9). Furthermore, it has an exceptional capacity for survival in 20% NaCl (10). *Listeria monocytogenes* has a wide host range including humans, as well as wild and domestic animals that typically become infected by the ingestion of food or feed that has been contaminated with *L. monocytogenes* (11). Fish is a highly nutritious food that is widely marketed and consumed as a result of containing proteins with biological value and digestibility as well as lipids, mostly unsaturated fatty acids, vitamins, and minerals. Food that has been contaminated is the primary means of *Listeria* transmission in about 99% of instances (12). *L. monocytogenes* in fishes is likely from polluted waterways or during handling and processing with contaminated environments and equipment (13,14). The bacterium has frequently been isolated from fish and fish products acting as vehicles worldwide (15,16). Vegetables have also been noted as a potential cause of listeriosis for humans; this can occur directly through consuming contaminated raw vegetables or indirectly through consuming milk from animals that have ingested contaminated fodder or silage. In the last decade, there has been a significant increase in studies reporting the presence of *L. monocytogenes* in ready-to-consume fruits and vegetables. The prevalence of this bacterium has been estimated to range from 0.04% to 36.8% in various products, including cabbage, strawberries, cauliflower, celery, cucumber, carrots, herbs, lettuce, and cucumber (17). Globally, invasive listeriosis in humans is responsible for 20-30% of fatal cases. This condition is characterized by the invasion of the brain, bloodstream, or placenta, resulting in symptoms such as encephalitis, meningitis, and septicemia (18). Invasive listeriosis is commonly observed in immunocompromised people, neonates, older individuals, and pregnant women. However, it can occasionally affect healthy individuals. Many risks are related to the organism's existence during pregnancy, which may be asymptomatic or linked to vaginal infection in females, including the possibility of stillbirth, abortion, or significant neonatal complications. However, milder forms of listeriosis can result in symptoms such as nausea, persistent fever, vomiting, and flu-like illnesses, including chills, exhaustion, and muscle and joint pain (19). Essential genes must be present in the *L. monocytogenes* genome to cause infections in humans and animals and to be expressed under the appropriate conditions. Listiriolysine O (LLO) and internalin, which are expressed by the *hlyA* and internalin (*inl*) genes, respectively, are among the several virulence factors present in *L. monocytogenes* (20). The *hly* gene is involved in how *L. monocytogenes* controls the host cell. *InlA* may be involved in *L. monocytogenes'* penetration of intestinal epithelial cells by expressing the E-cadherin receptor. The *inlA* fragment contributes to *L. monocytogenes*' invasiveness and might distinguish invasive bacteria from noninvasive ones (21). Antibiotic resistance among foodborne bacteria, like *Listeria monocytogenes*, has been considerably enhanced by the increased use of antibiotics in recent years, which has occasionally led to their abuse in humans and animals (22). Recently, antibiotic resistance has increased in strains of *L. monocytogenes* from foodstuffs, the environment, and clinical samples., particularly those frequently employed to treat listeriosis. The resistance development among strains is usually affected by antibiotic usage in animals and people and geographic differences. Most *L. monocytogenes* strains are inherently resistant to cephalosporins and fluoroquinolones of the third and fourth generations currently in use, which may negatively impact human health (23). Fast pathogen identification in food products is essential for the diagnosis of food illness and for monitoring food safety (24).

Consequently, the current research sought to assess the presence of *L. monocytogenes* in fish, vegetables, and humans in Qena Governorate, Egypt, using both conventional and molecular approaches, in addition to the virulence gene profile, antimicrobial sensitivity and phylogenetic analysis of the recovered isolates.

Materials and methods

Ethics approval and consent to participate

The South Valley University ethical committee in Qena, Egypt, approved this study (No. 8b/16.01.2021). Additionally, each participant's agreement had been gotten orally.

Study design and sampling

This research was carried out in Qena Governorate of Egypt, which is located approximately 576 km from Cairo, between February 2021 and November 2022. The entirety of 345 different samples were gathered and investigated. Our samples included 115 fish, 25 swabs from fish containers, and 90 vegetable samples obtained randomly from various markets and supermarkets in Qena, Egypt, and were

immediately transported in ice boxes to the laboratory for bacteriological analysis.

For each fish sample, 25 grams of the interior flesh content, including the freshly caught and frozen fish slime and 25 grams from every vegetable, were mixed for 2 minutes in 225 ml of *Listeria* selective broth base (HiMedia, M889, India). The collected fish containers were swabbed using sterile cotton swabs. Then they put in screw-capped tubes with 5 ml of *Listeria* selective broth base (HiMedia, M889, India). On the other hand, 90 samples of diarrhea from patients admitted to clinical labs in Qena with gastrointestinal issues and diarrhea were used in the human survey. The age, sex, and residence of each patient were recorded. In addition, 25 hand swabs were collected from fishermen. The fecal and hand swabs were collected in sterilized screw-capped tubes with 5 ml of *Listeria* selective broth base.

Listeria monocytogenes isolation and identification

L. monocytogenes was isolated by samples were pre-enriched for *Listeria* species by incubation in *Listeria* selective broth base (HiMedia, M889) with *Listeria* Selective Supplement II, modified (HiMedia, India) at 35°C for two days (25). A loopful from the *Listeria* Selective Broth Base culture was used to streak on Oxford Medium Base (HiMedia, M1145, India) with Oxford *Listeria* supplement (HiMedia, Modified FD172, India) followed by incubation at 35°C for two days. Five potential colonies were cultured on tryptone soya agar (Himedia, M291, India) plus 0.6% yeast extract (TM MEDIA) and incubated at 37°C overnight. The purified colonies were identified biochemically by performing a gram staining, catalase procedure, β-hemolytic activity, motility procedure at 25°C and 37°C, and CAMP procedure (26).

DNA extraction

The ABT bacterial DNA Mini Extraction Kit was employed to obtain genomic DNA from *L. monocytogenes* cultures following the guidelines provided by the manufacturer (spin column) (cat. no. ABT001). The obtained DNA was maintained at -20°C till it was needed.

PCR amplification

In this study, the primers used were obtained from Metabion (Germany); according to Dalmasso *et al.* (27) and Kumar *et al.* (28) and two additional primers were designed in this study (Table 1). The experimental protocol involved adding 1 µl of each 10 pmol primer to a 25 µl reaction mixture consisting of 12.5 µl of WizPure PCR 2X Master (W1401), 5 µl of DNA template, and 5.5 µl of nuclease-free water (Qiagen, Germany). The reactions were performed under different conditions for each primer set as follows: primary denaturation at 94°C for 5 min followed by 35 cycles of 94°C-30 sec denaturation for all primers, while 55°C-40 sec annealing, 72°C-45 sec extension, and 72°C-10 min final extension for *Listeria 16S rRNA* (437 bp); 60°C-40 sec annealing, 72°C-1.2 min extension, and 72°C-12 min final extension for *Listeria monocytogenes 16S rRNA*(1200 bp); 59°C-30 sec annealing, 72°C-30 sec extension, and 72°C-10 min final extension for *inlA* gene (380 bp); 58°C-30 sec annealing, 72°C-45 sec extension, and 72°C-10 min final extension for *hlyA* gene (501 bp). One hundred milliliters of TBE buffer made 1.5% agarose gel. Five µl of DNA ladder (100 bp ladder, Diagnostic Biotech, M1060) and ten µl of negative control, positive control, and PCR product were loaded onto the gel. Electrophoresis was carried out at 80 volts for 45 minutes using a power supply (Cleaver Scientific Ltd., NanoPAC-300, England). Finally, the gel documentation system took the result of the gel (Cleaver Scientific Ltd., England).

Table 1: Primers for the *L. monocytogenes* genes used in this investigation

Target gene	Primer sequence	References
Listeria 16S rRNA	CCTAATACATGCAAGTCGAA ACAAGCAGTTACTCTTATCCTT	27
L. monocytogenes 16S rRNA	GGACCGGGGCTAATACCgAATgAT AA TTC ATG TAG GCG AGT TGC AGC CTA	28
*inl*A	CAGGCAGCTACAATTACACAAGA AGCGGGTCTATATCCGTTATCT	Our study
*hly*A	ACCTACAAGACCTTCCAGATTTTTC GCAACGTATCCTCCAGAGTG	

Phenotypic detection of antibiotic resistance

Antibiotics were selected based on their widespread use and availability in the investigation areas for both animal and human treatment. The antimicrobial resistance test was conducted using the disc diffusion approach on Muller-Hinton agar plus 5-10% sheep RBCs, following the National Committee for Clinical Laboratory Standards (29) guidelines. This study evaluated the susceptibility of eight antibiotics (Oxiod®) at the following concentrations (µg/ml): gentamicin (10), chloramphenicol (30), amoxicillin-clavulanic acid (10), tetracycline (30), enrofloxacin (5), trimethoprim-sulphamethoxazole (25), ciprofloxacin (10), and ampicillin (10). According to Singh *et al.*'s (30), each isolate's multiple antibiotic resistance (MAR) index has been estimated.

Sequencing of *Listeria 16S rRNA* gene and Phylogenetic analysis

The 16S rRNA gene PCR products for seven *Listeria monocytogenes* isolates, including one each from fishermen's hand swabs (HS5), cauliflower (VC4), tilapia (TF1), catfish (CF2), and fish containers swabs (FC3), as well as two isolates from diarrheal swabs (DS6 and DS7), were purified employing the QIA quick extraction kit (Qiagen, Valencia, CA). The Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used to sequence the resulting refined products employing an Applied Biosystems 3130 Sequencer (ABI, USA). The sequence reaction was purified using Centrisep (spin column) according to the instructions for Cat. No. CS-901 of 100 reactions; To identify the sequences and assign them to GenBank, an initial analysis was conducted employing the Basic Local Alignment Search Tool (BLAST®) (31). The resulting sequences were then submitted through BLAST similarity and phylogenetic analysis employing the neighbor-joining method through the Mega 6 program (32).

Statistical analysis

the data were statistically analyzed by using SPSS version 22. The relationship between variables has been established using Fisher's exact and Monte Carlo tests. Fisher's exact test evaluated the null hypothesis of independence. At the same time, Monte Carlo simulation modeled the possibility of several outcomes in a process that was challenging to anticipate because it involved random variables. A *p*-value of less than 0.05 was regarded as statistically significant for the variations in prevalence rates. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated for every variable. The diagnostic accuracy approach assessed the degree of agreement with the two diagnostic techniques used through the McNemar test.

Results

Occurrence of *Listeria monocytogenes* in various source

Table 2, figures 1 and 2 present data indicating that the occurrence of *Listeria monocytogenes* in this study was substantially greater when detected using the conventional method (11.30%, obtained from 39 out of 345 samples) compared to the PCR test (3.48%, obtained from 12 out of 345 samples). The occurrences of *L. monocytogenes* in fish, fish containers, and vegetables were 8.70 and 3.48, 20 and 8, and 18.89 and 1.11% by conventional method and PCR, respectively. On the other hand, 6.09% and 4.35% of the samples obtained from humans tested positive for *Listeria monocytogenes* using conventional methods and PCR tests, respectively. The detection of *L. monocytogenes* and the source of the tested samples were significantly associated (Figures 1 and 2).

From Table 3, it is certain that fresh fish harbor *Listeria monocytogenes* at 12.31% and 6.15% by conventional method and PCR, respectively. Fresh fish of two species were investigated, tilapia and catfish, with the acquisition of *L. monocytogenes* occurrence in tilapia and catfish as 5 and 8%, respectively. In contrast, the traditional approach revealed a 4% (2/50) incidence of *L. monocytogenes* in frozen fish, which PCR could not confirm. A statistically insignificant difference was found between *L. monocytogenes* incidence in fresh and frozen fish (value = 3.809; $p = 0.113$).

Regarding the vegetable samples, *L. monocytogenes* was detected in parsley and lettuce at 26.67% and 13.33%, respectively, using the conventional method, but PCR could not confirm it. In the case of cauliflower, *L. monocytogenes* was detected in 5 samples (16.67%) using the conventional method. Still, PCR confirmed only one sample (3.33%). However, the association between the results obtained by the two methods was statistically insignificant ($\chi 2 = 1.84$; $p = 1.000$) (Table 4).

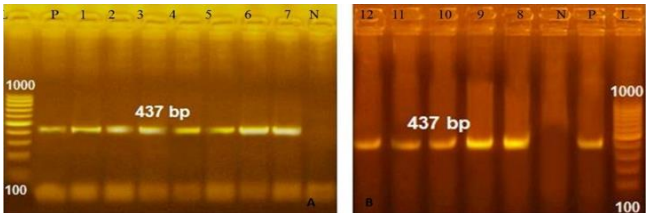

Figure 1: Agarose gel electrophoresis of *Listeria 16S rRNA* gene showing clear bands at 437 bp L: 100 bp ladder, P: positive control, N: negative control, lane 1 and 2 isolates from tilapia, lane 3 and 4 isolates from catfish, lane 5 and 6 isolates from fish containers, lane 7 isolate from cauliflower, lane 8-11 isolates from diarrheal samples, lane 12 isolate from hands of fishermen.

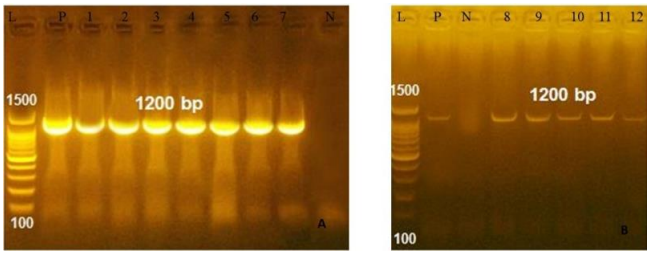

Figure 2: Agarose gel electrophoresis of *L. monocytogenes* 16S *rRNA* gene showing clear bands at 1200 bp L: 100 bp ladder, P: positive control, N: negative control, lane 1 and 2 isolates from tilapia, lane 3 and 4 isolates from catfish, lane 5 and 6 isolates from fish containers, lane 7 isolate from cauliflower, lane 8-11 isolates from diarrheal samples, lane 12 isolate from hands of fishermen.

Table 2: Occurrence of *Listeria monocytogenes* in the investigated samples using conventional method and PCR test

Sources	No. examined	+ve *L. monocytogenes* No. (%)		X^2 (*P*)	Odds ratio
		Conventional methods	PCR		
Fish	115	10 (8.70)	4 (3.48)	0.000*	1.261(0.33-4.822)
Fish container	25	5 (20)	2 (8)		0.523(0.095-2.862)
Vegetables	90	17 (18.89)	1 (1.11)		4.045(0.464-35.259)
Human	115	7 (6.09)	5 (4.35)		References
Total	345	39 (11.30)	12 (3.48)		

* The significant level at P<0.05.

Table 3: Occurrence of *Listeria monocytogenes* in the investigated fish samples using conventional method and PCR test

Sources	No. examined	+ve *L. monocytogenes* No. (%)		Monte Carlo X^2 (*P*)	Odds ratio
		Conventional methods	PCR		
Tilapia (Fresh)	40	4 (10)	2 (5)	3.809 (0.113)	0.61(0.08 -4.6)
Catfish (Fresh)	25	4 (16)	2 (8)		
Frozen fish	50	2 (4)	0 (0)		
Total	115	10 (8.70)	4 (3.48)		

Table 4: Occurrence of *Listeria monocytogenes* in the investigated vegetable samples using conventional method and PCR test

Sources	No. examined	+ve *L. monocytogenes* No. (%)		Monte Carlo X^2 (*P*)	Odds ratio
		Conventional methods	PCR		
Parsley	30	8 (26.67)	0 (0)	1.84 (1.000)	NA
Lettuce	30	4 (13.33)	0 (0)		
Cauliflower	30	5 (16.67)	1 (3.33)		
Total	90	17 (18.89)	1 (1.11)		

Occurrence of *L. monocytogenes* in humans

Table 5 indicates that *L. monocytogenes* was isolated from hand swabs of fishermen using the conventional method with a percentage of 20%, which was higher than that detected by PCR 4%. However, both methods detected *L. monocytogenes* in diarrheal samples with the same percentage of 4.44%. Fishermen and diarrheal people infected with the organism did not differ significantly from one other (p = 0.509, odd ratio 0.061-5.601).

A total of 90 diarrheic patients (35 men and 55 women) between the ages of 5 and 56 years participated in this study. Patients aged 5 to 17 years and 31 to 43 had the highest incidences of *L. monocytogenes* 5.71% and 5.26%, respectively, followed by patients aged 18 to 30 (3.70%). However, patients aged 44 to 56 showed no evidence of the infection. *Listeria monocytogenes* was more common in females 7.27%, 4 out of 55 than in males who did not have the infection. Furthermore, *L. monocytogenes* was more frequent in individuals residing in urban areas 4.76% than in rural areas 3.70%. However, the difference was not statistically significant (Table 6).

Table 7 demonstrate the occurrences of *L. monocytogenes* virulence factors, where the *inlA* virulence gene was found in all 12 isolated strains. In contrast, only five isolates contained the hlyA virulence gene, distributed as two fish isolates and one isolate each from fish containers, vegetables, and humans (Figures 3 and 4).

Table 5: Occurrence of *Listeria monocytogenes* in the investigated human samples using conventional method and PCR test

Sources	No. examined	+ve *L. monocytogenes* No. (%)		Fisher's Exact Test	Odds ratio
		Conventional methods	PCR		
Faecal	90	4 (4.44)	4 (4.44)	0.509	0.583(0.061-5.601
Hand	25	5 (20)	1 (4)		
Total	115	7 (6.09)	5 (4.35)		

Antibiotic resistance profile of *L. monocytogenes*

Table 8 presents the antimicrobial resistance profile of *Listeria monocytogenes* isolates, with all isolates exhibiting resistance to ampicillin 100 and 50% of the isolates being gentamicin resistant. The highest susceptibility levels were observed for enrofloxacin 100%, followed by amoxicillin-

clavulanic acid and chloramphenicol 91.67% each, and a moderate level of sensitivity to tetracycline 75%. The highest MAR value of 0.625 was recorded for an *L. monocytogenes* isolate obtained from a diarrheal swab (Table 9). The mean MAR index for *L. monocytogenes* isolates was 0.271, ranging from 0.125 to 0.625. Among our isolates, 25% had a MAR value of less than 0.2, whereas 75% had a MAR index higher than 0.2. Moreover, 3 out of 12 *Listeria monocytogenes* isolates 25% have been identified to be multidrug resistant.

Table 6: Occurrence of *L. monocytogenes* in human samples according to age, sex, and residence using the PCR test

Variables	No. examined	+ve *L. monocytogenes* No. (%)	Monte Carlo Value (*P*)	Odds ratio
Age (year)				
5-17	35	2 (5.71)	2.304(0.502)	4.143 (0.332-51.764)
18-30	27	1(3.70)		3.71 (0.205-67.149)
31-43	19	1(5.26)		2.714 (0.149-49.533)
44-56	9	0(0)		Reference
Sex				
Males	35	0(0)	1.000	0.559 (0.060-5.188)
Females	55	4(7.27)		
Residence				
Urban	63	3(4.76)	1.000	1.250 (0.201-7.778)
Rural	27	1(3.70)		

Table 7: Occurrence of some virulence genes in Listeria monocytogenes isolates obtained from the examined samples

Sources	No. isolates	*inl*A gene No. (%)	*hly*A gene No. (%)	Monte Carlo Sig
Fish	4	4(100)	2(50)	0.9444 (0.814691)
Fish container	2	2(100)	1(50)	
Vegetables	1	1(100)	1(100)	
Human	5	5(100)	1(20)	
Total	12	12(100)	5(41.67)	

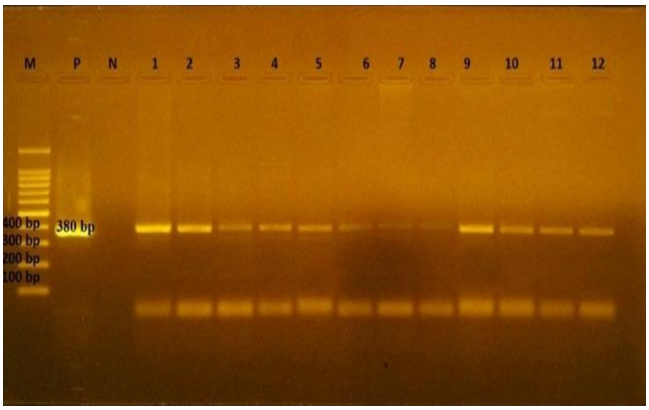

Figure 3: Agarose gel electrophoresis of *L. monocytogenes* for detection of *inlA* virulence gene showing clear bands at 380 bp. M: 100 bp ladder, P: positive control, N: negative control, lane 1 and 2 isolates from tilapia, lane 3 and 4 isolates from catfish, lane 5 and 6 isolates from fish containers, lane 7 isolate from cauliflower, lane 8-11 isolates from diarrheal samples, lane 12 isolate from hands of fishermen.

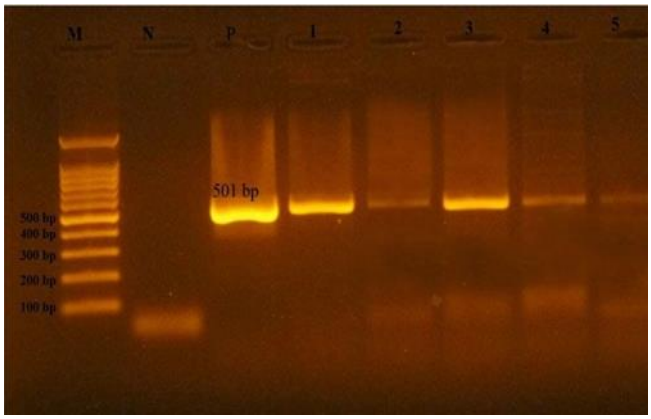

Figure 4: Agarose gel electrophoresis of *L. monocytogenes* for detecting *hly*A virulence gene showing clear bands at 501bp. M: 100 bp ladder, P: positive control, N: negative control, lane 1 and 2 isolates from catfish, lane 3 isolate from the fish container, lane 4 isolate from cauliflower, lane 5 isolate from hands of fishermen.

Table 8: Antimicrobial resistance of *Listeria monocytogenes* strain

Antimicrobial agents	Resistant No. (%)	Intermediate No. (%)	Susceptible No. (%)
Gentamycin	6(50)	1(8.33)	5(41.67)
Chloramphenicol	0(0)	1(8.33)	11(91.67)
Amoxicillin-clavulanic acid	1(8.33)	0(0)	11(91.67)
Tetracycline	3(25)	0(0)	9(75)
Enrofloxacin	0(0)	0(0)	12(100)
Ciprofloxacin	1(8.33)	5(41.67)	6(50)
Sulfamethoxazole-trimethoprim	3(25)	1(8.33)	8(66.67)
Ampicillin	12(100)	0(0)	0(0)

Table 9: Antimicrobial resistance profile of *Listeria monocytogenes* isolates

No.	Sources of isolates	Antimicrobial resistance profile	MAR index
1	Hand swab	AM	0.125
2	Fish container swab	AM	0.125
3	Fish container swab	TE, AM	0.25
4	Tilapia	CN, SXT, AM	0.375
5	Tilapia	AM	0.125
6	Catfish	TE, AM	0.25
7	Catfish	CN, AM	0.25
8	Vegetable	SXT, AM	0.25
9	Diarrheal swab	CN, AM	0.25
10	Diarrheal swab	CN, AM	0.25
11	Diarrheal swab	CN, AMC, TE, CIP, AM	0.625
12	Diarrheal swab	CN, SXT, AM	0.375
Average 0.271			

Phylogenetic analysis

Seven strains of *Listeria monocytogenes* were subjected to phylogenetic and sequence analyses of the *16S rRNA* gene. The findings demonstrated that the tested isolates shared a striking genetic similarity with other *L. monocytogenes* strains from various sources submitted to the GenBank database. These sequences, with the accession numbers OQ024047, OQ024048, OQ024049, OQ024050, OQ024051, OQ024052, and OQ024053, were uploaded to the GenBank. They were isolated from swabs taken from fishermen's hands, cauliflower, tilapia, catfish, and fish containers, as well as two isolates from diarrheal swabs, respectively (Figures 5).

Phylogenetic and sequence analyses of the *16S rRNA* gene illustrated that our examined strains demonstrated a complete similarity 100% to the *Listeria monocytogenes* strain isolated from caprine in the USA with accession No. CP062127. Furthermore, they displayed great similarity rates with *Listeria monocytogenes* strains isolated from beef sausage in the USA 99.8%, cheese in Austria 99.8%, patients with cystic fibrosis in Canada 99.1% with the accession numbers CP068977, FR733650, and EU090894, respectively.

Our seven *L. monocytogenes* strains were found to be clustered together with other *Listeria* species in the phylogenetic tree, including *L. welshimeri* and *L. seeligeri*, which were recovered from food processing facility and raw milk having accession codes of KC441000 and DQ065844, respectively. While an apparent diversity was found with some other *Listeria* species, such as *L. thailandensis* with accession No. NR-180224, isolated from fried chicken in Thailand (Figure 5).

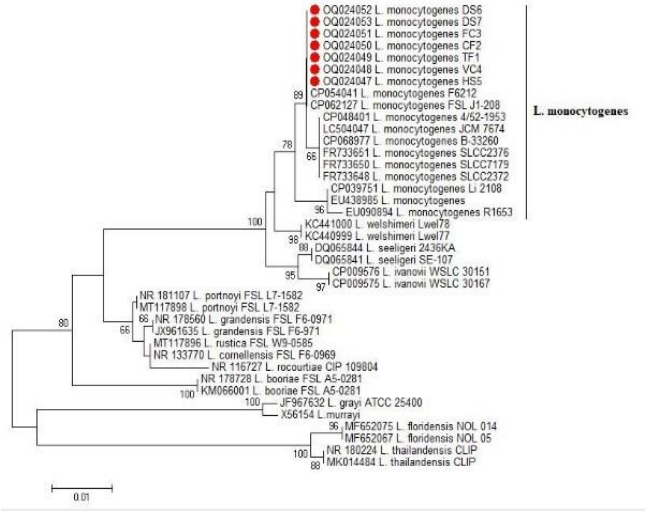

Figure 5: The phylogenetic analysis of the *16S rRNA* gene from the seven *Listeria monocytogenes* strains isolated from various origins. The phylogenetic tree shows the genetic matches between the stains under investigation and those submitted to the GenBank database.

Discussion

Various diseases and fatalities can be brought on by foodborne microorganisms globally (33). The bacterium *Listeria monocytogenes* is a significant foodborne organism associated with numerous serious outbreaks; moreover, it may contaminate food items during or after processing. In the present study, *L. monocytogenes* varied among various sources, with a total incidence of 11.30 and 3.48% using

conventional and PCR procedures, respectively. It is possible that the higher incidence rate observed with the conventional method was due to false-positive colonies, as reported by Kim *et al.* (34).

Various investigations have recorded different incidence rates of *L. monocytogenes*. Yan *et al.* (8), Abdollahzadeh *et al.* (35), Al-Gburi (36), Bouymajane *et al.* (37), and Elsayed *et al.* (38) reported prevalence rates of 4.13, 2.95, 6.66, 2.9, and 11.9%, respectively. The variation in incidence rates observed in many investigations might be due to differences in sample sizes, isolation techniques, and geographical locations, as suggested by Diriba *et al.* (39), which is similar to our opinions.

The environment and equipment used at various processing phases are reliable sources for tracing *Listeria* contamination in the seafood sector (40). In this study, the highest incidence of *L. monocytogenes* 8% was identified among fish containers, which agrees with the results of Pedro *et al.* (41) at 7.20%. However, a lower percentage 1.1% was recorded (31), and a higher percentage also have been recorded 17.1% (42), while Abdollahzadeh *et al.* (35) could not detect the organism in fish containers. The current study's highest contamination rate for fish containers could be explained by the tendency of *L. monocytogenes* to attach and survive on environmental surfaces, especially in insufficient or incomplete cleanliness and sanitation (43). In addition, the formation of biofilms makes *Listeria monocytogenes* more resistant to antimicrobials and sanitizers (44).

Fish is the main source of animal protein for about 1 billion people, so it has become an essential part of diets globally (45). However, at the same time, it is a potential source of *Listeria* infection. According to Food Safety News, many researchers have tracked 22 outbreaks of listeriosis between 2010 and 2021 that were probably related to consuming smoked, gravlax, and other fish products. *L. monocytogenes* tolerance levels are zero for frozen and fresh fish under Egyptian food safety laws (46). In the present investigation, *L. monocytogenes* was found in the examined fish's 3.48% (4/115), with 6.15% (4/65) detected in fresh fish samples. In contrast, it could not be detected in frozen fish. A similar incidence of fresh fish 6% (47), a higher incidence of 19.3% was also recorded (48). On the other hand, lower percentages were obtained at 0.2% and 4.28% in Turkey (49) and Jordan (50), respectively. Fish can become infected with *Listeria* in one of two ways: direct contact with polluted water or ingesting bacteria via contaminated feed or sediments. Consequently, the microorganisms detected in fish could be used to infer the health and safety of aquatic habitats (51).

Our study evaluated the presence of *Listeria monocytogenes* in two globally renowned fish species, tilapia and catfish. Furthermore, these species are popular traditional foods in Egypt and have been previously considered possible sources of *Listeria monocytogenes* (52). The results showed that the frequency of *L. monocytogenes* was 5% in tilapia and 8% in catfish. A comparable result was reported by El-Demerdash *et al.* (53) for tilapia in Egypt 6%. However, a relatively higher isolation rate of 33.3% was recorded in raw Tilapia in Iran by Abdollahzadeh *et al.* (35). Nevertheless, the absence of *Listeria monocytogenes* in Nile tilapia samples marketed in Zagazig, Sharkia governorate (54).

Chen *et al.* (52) in the USA recorded the isolation of *L. monocytogenes* with high percentages 43.3 and 76.7% in fresh unchilled and chilled catfish fillets, respectively, but could not isolate it from catfish skins and intestines. In contrast, an investigation by Abdallah-Ruiz *et al.* (43) conducted in the southeastern United States observed a lower percentage 2% of live catfish.

The current findings indicated a higher incidence of *L. monocytogenes* in catfish than tilapia, possibly because catfish are bottom eaters and pollutants tend to settle in their environment. Moreover, catfish are known for their scavenging behavior and ability to adapt to their surroundings, particularly in polluted water with hypoxia. As a result, they can withstand sewage, making them have greater potential for contamination by pathogenic bacteria like *L. monocytogenes*.

In this study, *L. monocytogenes* could not be isolated from frozen fish samples, following the finding of Rahimi *et al.* (15). Conversely, Tarazi *et al.* (50) recovered the microorganism in 6.6% of the examined frozen fish samples. These discrepancies in results could be attributed to differences in sampling techniques and analytical methods used. Additionally, various geographic regions and processing facilities may have varying levels of raw fish contamination, which can affect the incidence of *Listeria monocytogenes* (55).

In the current investigation, the lowest incidence of *L. monocytogenes* was found in vegetable samples 1.11% using PCR, consistent with the findings of Luchansky *et al.* (56), who recorded a similar incidence of 1.12%. While Maćkiw *et al.* (57) reported a lower incidence of 0.56 %. Conversely, Bouymajane *et al.* (37) failed to find *L. monocytogenes* in salads in Morocco, while Cordano and Jacquet (58) and Sant'Ana *et al.* (59) reported higher incidences of 15.3 and 3.1%, respectively.

Of the three types of vegetables investigated in our study, *L. monocytogenes* was only found in cauliflower at a rate of 3.33%. At the same time, parsley and lettuce were negative for the bacterium. Similarly, Cordano and Jacquet (58) could not recover *L. monocytogenes* from lettuce or cauliflower. However, Sant'Ana *et al.* (59) noted a higher prevalence of 2.0% in lettuce. The growth of *L. monocytogenes* in vegetable samples is affected by several variables, including the food's intrinsic qualities (such as water activity, NaCl content, pH, and associated microflora) and extrinsic variables (such as gas atmosphere and temperature) Sant'Ana *et al.* (60). In our research, *L. monocytogenes* was found only

in cauliflower. This finding may be related to the fact that cauliflower grows in fertile, well-drained, and moisture-retentive soil with a pH between 6 and 7, the conditions which facilitate the growth of *L. monocytogenes* (61).

Concerning human samples, similar occurrence rates of *L. monocytogenes* were detected in hand swabs of fishermen and diarrheal samples 4 and 4.44%, respectively using PCR. Abbasi *et al.* (62) reported a closely related incidence of 5.2% in diarrheal samples. However, Meghdadi *et al.* (63) and Abdelaziz and Mohamed (64) identified increased incidences of 7.5% and 12.5%, respectively. Ahmed *et al.* (54) documented a lower percentage 2% of *L. monocytogenes* in diarrheal samples, while Cheun *et al.* (65) was unable to isolate *L. monocytogenes* from human diarrheal samples. Our findings confirm that human wastes play a role in transmitting zoonotic pathogens (66). Fallah *et al.* (42) and Jamali *et al.* (13) identified variable incidences of *L. monocytogenes* in hand swabs from fishermen, with rates of 16.2% and 2.1%, respectively. Conversely, Akkaya *et al.* (67) could not isolate *L. monocytogenes* from the hand swabs of fishermen. Differences in the sensitivity of the enrichment, diagnostic, and culture procedures employed to identify the existence of *L. monocytogenes* may explain the variations in patient samples between our study and those reported in other studies. Variations in nutrition, resistance to *L. monocytogenes* infection, and level of exposure to environmental *L. monocytogenes* bacteria reservoirs may also exist (68). A retrospective study in Finland (69) revealed the risks of consuming seafood contaminated with *L. monocytogenes*. According to a Finland study, a strain of *L. monocytogenes* detected in multiple sporadic cases of listeriosis was identical to an epidemic strain from fish.

Regarding age, two of the four *L. monocytogenes* strains 5.71% recovered from diarrheal samples were detected in individuals aged 5-17 years in our study, followed by one isolate each in the 31-43 (5.26%) and 18-30 (3.70%) year age groups. In research by Abbasi *et al.* (62), most L. monocytogenes infections 77.7% were noted in children under five.

In the present investigation, all patients with *L. monocytogenes* infections were females, while male patients did not have the infection. In an Iranian study by Abbasi *et al.* (62), male patients made up 55.5% of the *L. monocytogenes* infection patients, while female patients made up 44.4%. In Sweden, male patients comprised 47.9% of cases, while female patients comprised 52% (70). Females are more prone to contracting *L. monocytogenes* because they handle fish, which serves as a vehicle for *L. monocytogenes*. Moreover, they handle rabbits and poultry, such as fowl, turkeys, ducks, geese, pigeons, and others, which are sources of infection.

Regarding the residence risk factor, the incidence of *L. monocytogenes* in urban areas 4.76% was greater than in rural areas 3.70%. As the level of human activity and *L. monocytogenes* prevalence are correlated (51), the higher incidence in urban areas could be attributed to the higher population density and associated human activities. As well as food habits, people in urban areas usually eat ready-to-eat food from fish restaurants.

Although *L. monocytogenes* is not the main source of foodborne illness, it has the greatest rate of fatalities due to certain virulence characteristics (71). In our research, the occurrence of the *inlA* gene, which is a bacterial surface protein for host cell attachment (72), was 100% in all *L. monocytogenes* strains noticed in fish, fish containers, vegetables, and human samples. The present findings are consistent with prior investigations in which *L. monocytogenes* samples were recovered from diverse sources, such as fish (49), fish containers (13), vegetables (57), and humans (63), and were found to harbor the *inlA* gene. However, Abdelaziz and Mohamed detected a comparatively lower prevalence 33.3% of the gene in human isolates (64).

LLO, a pore-forming surface toxin produced by the hemolysin A (*hlyA*) gene, is necessary to lysis vacuole membranes and release *L. monocytogenes* into the cytoplasm (73). The overall incidence of this gene was 41.67%, distributed as follows: fish 50%, fish containers 50%, vegetables 100%, and humans 20%. While Jamali *et al.* (13) and Bouymajane *et al.* (37) detected the *hlyA* virulence-associated gene in all *L. monocytogenes* isolates in fish and both fish containers and humans, respectively, the same result in vegetables 100% was obtained by Soni *et al.* (74).

The emergence of antibiotic resistance is a further hazard to *L. monocytogenes* infections. This study discovered that ampicillin had the highest resistance rate 100%, followed by gentamicin 50%. Elsayed *et al.* (38) reported a resistance rate of 97.3% to ampicillin, which is close to our findings. Jamali *et al.* (13) reported a lower resistance rate 27.9% to ampicillin than our finding. On the other hand, Kuan *et al.* (75) indicated that their study detected no resistance to ampicillin. The frequent use of this antibiotic to treat infections in individuals and animals may account for the study's high resistance rate to ampicillin.

The appearance of gentamicin-resistant *L. monocytogenes* is becoming increasingly evident, as has been observed by our research and other investigators (76). In our study, all *L. monocytogenes* isolates noticed in human diarrheal samples were resistant to gentamicin, which might be attributed to the overuse of aminoglycosides in human medicine. Meanwhile, the highest sensitivity levels in this study were recorded for enrofloxacin 100%, amoxicillin-clavulanic acid 91.67%, chloramphenicol 91.67%, and tetracycline 75%. Moreover, Lotfollahi *et al.* (77) and Çokal *et al.* (78) mentioned that *L. monocytogenes* isolates had complete sensitivity 100% for amoxicillin-clavulanic acid, tetracycline, and chloramphenicol, respectively. In contrast, Akrami-Mohajeri *et al.* (79) indicated no sensitivities were detected to tetracycline, chloramphenicol, or amoxicillin/clavulanic acid.

In addition, 25% of *L. monocytogenes* recovered isolates showed multidrug resistance. This is a significant public health issue since it might make treating listeriosis more difficult. While the lower occurrence obtained by Garedew *et al.* (47) was 16.7%, in contrast, Akrami-Mohajeri *et al.* (79) and Elsayed *et al.* (80) mentioned that all their *L. monocytogenes* strains exhibited multidrug-resistant.

The current data show that the MAR index for *L. monocytogenes* isolates ranged from 0.125 to 0.625. Badawy *et al.* (81) observed a greater MAR index 0.33 to 0.88 for *L. monocytogenes* identified in dairy cattle, the environment, and dried milk in Egypt. In contrast, lower MAR indices 0.07 to 0.50 were detected in milking equipment, raw milk, and dairy workers by Tahoun *et al.* (76), and 0.17- 0.42 were detected in animals, humans, and animal feed by Elsayed *et al.* (38), respectively.

In the current research, MAR indices higher than 0.2 were present in 75% of *L. monocytogenes* isolates, with the most significant MAR index 0.625 detected in the *L. monocytogenes* isolate noted in a human diarrheal swab, indicating a high-risk source of contamination in which antibiotics were overused (81). In contrast, MAR indices of less than 0.2 were present in only 25% of isolates, indicating that these isolates came from a location where antibiotics are not commonly employed (82).

Because food-borne *L. monocytogenes* strains are becoming increasingly antibiotic-resistant, future outbreaks may be challenging to control (83). Additionally, no commercially available vaccination is available to protect against listeriosis (84).

The primary source of phylogenetic data for microorganisms, 16S rRNAs (a recognizable marker for a bacterial species), are currently used for microbial characterization and classification. Further, numerous studies have demonstrated the important role of 16S rRNA in the identification, differentiation, genetic relatedness, and phylogenetic analysis of *L. monocytogenes* from various environmental, clinical, and dietary samples (85). When investigating different sources and geographical areas, all *Listeria* 16S rRNA gene sequences were similar to prior sequences added to the GeneBank database. The seven bacterial isolates have a single common ancestor, as shown by the phylogenetic tree, which clarifies the imperative role that fish and vegetables play in transmitting human listeriosis. Nearly similar results were recorded by Soni *et al.* (74), and Abdeen *et al.* (86), both revealed great similarity (more than 99% nucleotide match). In the 16S rRNA gene sequences, we did not find any recent mutations which support the gene's slow evolution.

Conclusion

Vegetables and fish are a primary cause of *L. monocytogenes* infections in people, which has major global health implications. The severity of this disease has been compounded by widespread antimicrobial resistance, which increases the probability that treatment may fail by using available medications. Consequently, we urge all national health organizations to adopt stricter measures to prevent this resistant pathogen from developing.

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Conflict of interest

The authors affirm that they do not have any conflicts of interest.

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مدي انتشار ومقاومة المضادات الحيوية وتحليل النشوء والتطور لليستيريا مونوسيتوجين المعزولة من مصادر مختلفة في مصر: الأسماك والخضروات والبشر

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الخلاصة

تعتبر الاصابة بجراثيم الليستيريا عدوى شديدة تنتج عن تناول طعام ملوث بهذه الجراثيم والتي تعتبر المسبب الرئيسي للإصابة بهذا المرض في الإنسان. وهدفت هذه الدراسة إلى التعرف على النمط الظاهري والوراثي لليستيريا مونوسيتوجين عن طريق استخدام جينات *inlA* و *hlyA*، متبوعة بتحليل تسلسل الحمض النووي وحساسية مضادات الميكروبات في المواد الغذائية والعينات البشرية. وقد تم الحصول على العينات بشكل عشوائي من مختلف الأسواق ومحلات السوبر ماركت في قنا بمصر، وتتضمن ١١٥ سمكة، و ٢٥ حاوية أسماك، و ٩٠عينة خضار، و ٩٠ عينة إسهال، و ٢٥ مسحة يد من مرضى وصيادين على التوالي. وتم التأكيد على تواجد جراثيم الليستيريا مونوسيتوجين في ١١,٣٠٪ (٣٤٥/٣٩) و ٣,٤٨٪ (٣٤٥/١٢) من العينات التي تم فحصها باستخدام طرق العزل التقليدية وتفاعل البلمرة المتسلسل على التوالي. وكانت نسبة التواجد في الأسماك وحاويات الأسماك والخضروات والبشر ٨,٧٠، ٣,٤٨، ٢٠، ٨، ١٨,٨٩، ١,١١ ٤، ٦,٠٩ و ٤,٣٥٪ بالطريقتين على التوالي، مع وجود فرق ذي دلالة إحصائية. وعلى الرغم من تواجد ميكروب ليستيريا مونوسيتوجين بصورة سائدة في الفئات العمرية ٥-١٧ و٣١-٤٣ سنه، فقد تم تحديد عامل الخطر العمري للمرضى على أنه غير مهم إحصائيًا من منظور وبائي. وتم العثور على حالات أعلى في الإناث والمناطق الحضرية ٧,٢٧ و ٤,٧٦٪ منها في الذكور والمناطق الريفية ٠,٠٠ و ٣,٧٠٪، مع عدم وجود فرق معتد به إحصائياً. وتم العثور على جين *inlA* في جميع العزلات في حين أنه تم التعرف على جين *hlyA* في ٤١,٦٧٪. وتم استخلاص أعلى مؤشر مقاومة للمضادات الحيوية المتعددة ٠,٦٢٥ من مسحة الإسهال، وكانت جميع العزلات التي تم جمعها مقاومة تماما للأمبيسيلين. بالإضافة إلى ذلك، كانت ٢٥٪ من عزلات ليستيريا مونوسيتوجين مقاومة للأدوية المتعددة. وفقًا لتحليل النشوء والتطور، فإن العزلات المحلية التي تم الحصول عليها من عينات البلطي وسمك السلور وحاويات الأسماك والقرنبيط والبشر تشترك في الكثير من أوجه التشابه.

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